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We developed a simple method for estimating the real and imaginary refractive index of particles. This is done by filtering the particles (bacterial spores in our case) on a gold-coated filter membrane and performing IR microspectroscopy. The results are extremely reproducible (within particle counting statistics). A Kramers-Kronig consist iterative algorithm is applied to obtain scattering and absorption cross-sections for individual particles. The method was tested on spores and on polystyrene spheres.

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OPTICAL CONSTANT DETERMINATION OF BACTERIAL SPORES IN THE MIR

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Foreword

The primary objective of this research is to develop a new method for determining the optical constants of biological particles, focusing on bacterial endospores such as *B. subtilis*, a common simulant for anthrax. The purpose of the research is to provide a simple method for obtaining key information necessary for remote sensing of airborne bioparticles. ARO has enabled USC to perform calibration and sampling measurements and to begin comparing results obtained by our new method to those known in the literature using more complex sampling methods.

Samples of *B. subtilis* filtered across gold-coated filter membranes and were analyzed by reflectance microspectroscopy. Kramers-Kronig analysis of the mid-infrared reflectance spectra provided estimates of the real and imaginary parts of the complex refractive index. Using an iterative calculation, preliminary results show that it may be a practical possibility to calculate the wavelength dependence of absorption and scattering cross sections of this biological warfare stimulant. For comparison to materials with known optical properties, polystyrene microspheres deposited onto gold-coated anodiscs were also studied.

The method being developed has attracted the interest of pharmaceutical companies (Glaxo Smith Kline, Eli Lilly) who are interested in tools for studying powders more accurately than is presently possible with spectroscopy.

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Statement of the problem studied

Remote detection of bacterial spores and other particles by optical methods requires a careful analysis of the anticipated properties of these particles, particularly in the infrared and near-infrared spectral regions. Most analyses involve determining the scattering matrix elements for a particle type, but this calculation requires knowledge of the size and shape, as well as the real and imaginary refractive indices of particles.

The sizes and shapes and distributions of particles are relatively easily determined. However, the refractive index of particles is very difficult to obtain. The best measurements to date for bacterial spores have involved careful preparation of aerosols and fitting of observed behaviors to models. However, aerosol samples are constantly changing; it is difficult to know how many particles are being sampled, or what their state of aggregation in. Even when particle densities and aggregation states can be estimated, the estimations are never made on the actual sample being studied, but only on representative samples.

We developed a method for measuring a highly reproducible optical spectrum of particles that permits us to determine exactly what sample was measured. We have aimed to develop a method of relating the measured spectrum to the optical constants of the particles using Kramers-Kronig consistency as a major tool of the analysis.

Summary of the most important results

A. A Simple Method to Extract Optical Constants from Particulates by Infrared Reflectance Microscopy Demonstrated with Polystyrene Microspheres and *Bacillus subtilis* Spores

The potential threat of bioterrorism has grown in the last five years. This threat was realized in the fall of 2001, in which letters were sent via the US postal system containing *Bacillus anthracis*, resulting in 17 confirmed cases of inhalation or cutaneous anthrax. Of these cases, 5 proved fatal.[1] Due to the potential for some microorganisms to inflict harm to humans, particularly bacteria from the genera *Bacillus* and *Clostridium*, simple and reliable methods are required to aid in their detection and identification and thus curb the risk of exposure to biological warfare agents.[2]

Enumerating bacterial microorganisms in a liquid or aerosol sample can be accomplished by filtering them onto a membrane and then counting the number of organisms in a cross-sectional area. The use of filtration membranes such as polycarbonate and MF-Millipore[3-5] have been reported for filtering bacteria from solutions, soil or sediment. Additionally, cellulose-ester filter membranes have been used as growth media.[6] Enumeration of bacterial colonies in these studies was accomplished by staining of the bacteria with dyes and visible microscopy.[4,6] Fluorescence spectroscopy has also been used for the quantification of bacterial colonies on filtration membranes.[7-9]
FT-IR spectroscopic techniques provide both qualitative and quantitative chemical information simultaneously. Qualitative infrared research on different bacterial species on disposable polyethylene filter membranes has been reported.[10,11] Infrared microspectroscopy has been utilized in the study of heterogeneous organic particulate solids separated with Gold coated Nuclepore® polycarbonate membranes.[12]
A method of obtaining the optical constants of particulates using reflectance extinction spectra following their filtration on gold-coated porous alumina filters is presented here. This is demonstrated by the use of FT-IR spectroscopy and with scanning electron microscopy (SEM) in the study of polystyrene microspheres and *Bacillus subtilis* spores. Compared to organic membranes, alumina membranes have a highly ordered structure with a standardized pore sizes and are chemically inert.[13] Previously, bacterial strain discrimination by FTIR-NIR diffuse reflectance with an integrating sphere using alumina membranes has been reported.[14]

The filter media used for this study are based on Anodiscs™, porous alumina (Al_2O_3) substrates, which are then coated with an optically thick layer of gold to reflect infrared light, yet thin enough to prevent the closing of the substrate pores. The use of these filters for the mid-infrared studies of bacterial spores was previously demonstrated to be extremely statistically reproducible.[15] Using SEM to precisely enumerate the concentration of spores sampled in a 100 x 100 μm^2 area of the reflective filter media, a relative standard deviation of 0.0260 was calculated. Examination of the disc to disc variability for these substrates with FT-IR microscopy resulted in a low average relative

standard deviation (RSD) of 0.0482 for the Amide A band intensity at 3278 cm^{-1}. The filter methodology permitted a precise value for the extinction cross-section of individual spores as (7.8 ± 0.6) x 10^{-9} cm^2/endospore while the absorption cross-section was estimated as (2.10 ± 0.12) x 10^{-9} cm^2/endospore for the Amide A band.

THEORY

The most common expression for the transition of light through an absorbing media is transmittance:

$$T = \frac{I}{I_O} = \exp\left[-\sigma_{ext}\ N_{part}\ l\right] \tag{1}$$

where I_o and I, the intensities of the light from the source and through a sample respectively, are the measured quantities in single beam spectroscopy. The terms in the exponential expression are the numbers of particles per unit volume (N_{part}), path length l, and the total extinction cross-section (σ_{ext}). Similarly, for the work presented here, reflectance of particles on a surface can be expressed as:

$$R = \frac{I}{I_O} = \exp\left[-\sigma_{ext}\ N_{part}\right] \tag{2}$$

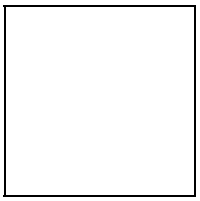
where the terms for the number of particles and path length are combined in N_{part} with units in # particles per unit area.

The total extinction cross-section is a sum of both the absorbance (σ_{abs}) and scattering (σ_{sca}) cross-sections and can be expressed in terms of Mie efficiencies (Q_{abs} and Q_{scat})[16]:

$$\sigma_{ext} = \sigma_{abs} + \sigma_{sca} = A\,(Q_{abs} + Q_{sca}) \tag{3}$$

where A is the geometric area of the particle (πr^2, where r is the particle's radius).

The absorbance and scattering efficiencies,[16] from Bohren and Huffman, are defined for particles small compared to the wavelength of light as:



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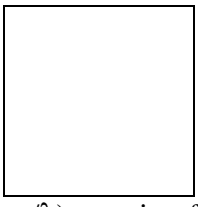
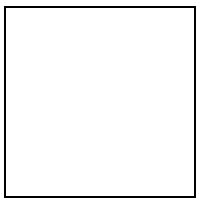


(5)

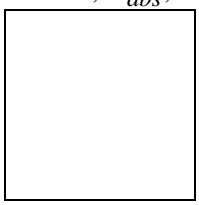
where x is referred to as the size factor ($2\pi r/\lambda$), a ratio of the particles size to the wave length (λ) of incident light. If the particle is sufficiently small, the term in brackets in Eq. 4 reduces to unity. The complex index of refraction (m) is a sum of the real (n) and imaginary (k) refractive indices ($m = n + ik$). The imaginary index of refraction is related to the extinction cross-section by:



(6)

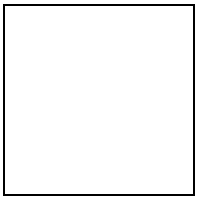
where c and ω are the speed of light and angular frequency respectively.

Because one cannot be sure how the size factor exactly influences σ_{abs} and σ_{sca}, in the reflection geometry, we can substitute a constant, C_{abs}, into Eq. 3 and express σ_{abs} by:



(7)

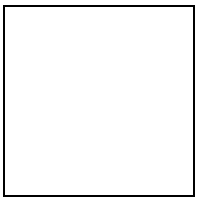
where C_{abs} is assumed to be of the order $8\pi^2 r^3$. Similarly, by factoring the wavelength term from the size factor in Eq. 5 and substituting a second constant, C_{sca}, for the remaining terms, we can express σ_{sca} by:



(8)

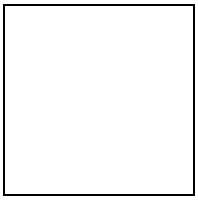
Here C_{sca} is assumed to be of the order $(128/3)\ \pi^5 r^6$. An alternate expression for the scattering constant (C_{sca}) in terms of the absorption constant (C_{abs}) is found, $C_{sca} \sim (2\pi/3)\ C_{abs}^{\ 2}$.

The quantitative relationship between a particle's real and imaginary refractive indices is given by the Kramers-Kronig transform. This dispersion relationship is well known and is written as:



(9)

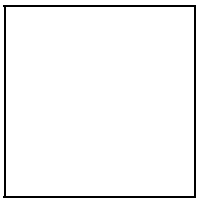
where P is the Cauchy principal part. However, because reflection measurements are obtained over a discrete wavelength or frequency (v) range, the Kramers-Kronig transform is more exactly written as:



(10)

If one knows either the real (n) or the imaginary portion (k) of the complex refractive index (m), one can utilize the Kramers-Kronig transform to compute the other part, and obtain the complex refractive index of a substance.[16]

Beginning with reflectance data, collected for example by FT-IR microscopy, and known values of how k varies with respect to wavelength, one can utilize an iterative fitting algorithm to obtain both constants C_{abs} and C_{sca} by:

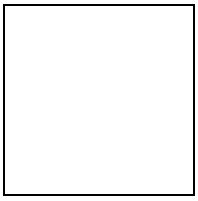


(11)

A second form of Equation 11 can be written in terms of only C_{abs} by substituting $2\pi C_{abs}/3$ for C_{sca}.

The number of particles can be found by SEM, while the Kramers-Kronig transform is used to calculate the complex index (m) from k. Once these constants are known a second iterative process is used to obtain the best estimate of the offset for the real refractive index (n_∞). Lastly, using Equations 7 and 8, the wavelength dependency of the absorbance and scattering cross-sections can be calculated. The cross-section information can then be used in quantitative analysis of future samples in order to estimate the number of particles that are deposited onto the reflective filter substrate. This is done by solving Eq. 1 for N_{part}.

By obtaining information about the absorbance and scattering constants of particles similar in nature and size, calculations can be made to extract information about bacterial endospores. A k spectrum is calculated to best fit the observed reflectance data by:



(12)

An alternative expression for the thickness can be used where l is replaced by the averaged volume to area ratio (vol/A) of the particle.

Spore Preparation and Filtration. Endospores of *Bacillus subtilis* strain PS832, a prototrophic laboratory strain derived from strain 168, were prepared at 37°C on 2xSG medium agar plates without antibiotics as described previously.[17] Although strain PS832 is derived from strain 168 which is the most commonly used laboratory stain, DNA sequencing demonstrates negligible differences between the two strains. The PS832 spore preparations were cleaned with numerous suspensions in water followed by centrifugation, as well as several sonication treatments until the preparations were free (> 98%) of sporulating cells, germinated spores or cell debris as determined by phase contrast microscopy.

A stock suspension of PS832 endospores was prepared by mixing 21.2 mg of the spore preparation with 50 mL of deionized ultrafiltered water (DIUF) (Fisher Scientific, Fairlawn, NJ). A 21.2 mg stock solution of polystyrene (PS) microspheres was prepared by diluting 0.424 g of a 5% w/v solution of 1 μm PS particles (Spherotech Inc., Libertyville, IL) with 50 mL of DIUF water. The solutions were prepared in amber bottles and were stored at 5 °C. Before filtration the solutions were sonicated for 15 min and then 0.50 mL portions were removed and diluted with 50 mL of DIUF water. These

solutions were sonicated for 10 min in order to minimize spore or PS microsphere aggregation. Following sonication, the solutions were filtered through Au coated Anodiscs™ (Whatman, Fisher Scientific).
The Anodiscs™ used were 47 mm in diameter with a nominal 0.2 μm pore size. They were coated with a ~100 nm Au layer using a CrC-100 sputtering system (Plasma Sciences Inc., Lorton, VA). The filter membranes were transferred to a vacuum filtration system with a holder for 47 mm filters (Fisher Scientific). The filter membranes were fragile, and care was taken to ensure that the samples studied here were free of cracks after filtering. To the vacuum filtration system was added the 50 mL bacterial spore or PS microsphere suspension. In each case, the prefiltered suspension was allowed to settle for approximately 5 min until the suspension ceased visibly moving. Vacuum filtration was then applied in order to achieve even distribution of individual spores throughout the surface of the Anodisc™ . After filtration, the disks were dried overnight in a compartment supplied with a dry air purge.

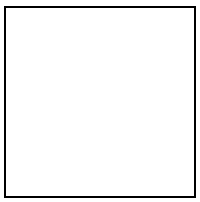
SEM and FT-IR Reflectance Microspectroscopy. After drying, the disks were cut into two nearly equal portions. One portion of the disk was used to collect images with an ESEM FEI Quanta 200 (FEI, Hillsboro, OR) in order to count the particles per unit area, while the other portion was used for microspectroscopy in the MIR region (650 - 4000 cm^{-1}). 10 images each were obtained by SEM at 8000x magnification of *B. subtilis* endospores and PS microspheres on the Au coated Anodisc™. The particles were counted in each image, recorded and extrapolated for an area equivalent to 100 x 100 μm^2, the area sampled by reflectance microscopy.

A Continuμm® microscope (with standard 15X, 0.58 NA infrared objective) coupled with a Nexus 470 FT-IR spectrometer using an MCT-A (mercury-cadmium-telluride) detector (Thermo Nicolet, Madison, WI) was used for reflectance microspectroscopy reported here. The spectra were collected in reflectance mode and were converted to absorbance through the OMNIC software, version 6.0a (Thermo Nicolet). The measurements were obtained at a resolution of 8 cm^{-1} using a triangular apodization function. The area sampled was adjusted to 100 x 100 μm^2 using a rectangular aperture. A background spectrum was obtained prior to each sample spectrum from a pre-cleaned gold-coated microscope slide due to their ease of use. Twelve extinction spectra of both the endospore sample preparation and polystyrene microspheres were collected, with 64 interferograms co-added and averaged, ensuring the microscope stage was positioned in different spots between each measurement. Data were imported into Matlab (version R14 Mathworks Inc. Cambridge Ma.) for analysis.

When particulates are filtered across a gold-coated alumina filter, using the methods described above, an even distribution is observed by SEM. This is illustrated in Figs. 1 and 2 illustrate this for *B. subtilis* spores and PS microspheres respectively. Using these reflective filter media, no aggregation is observed on the relatively flat filter surface. This enhances the reproducibility of this method compared to others, such as drying the suspensions directly onto a reflective media.[15] SEM can then be utilized for precise quantification of the number of particles deposited on these filters per unit area to be used in subsequent calculations. The averaged number of PS microspheres and *B. subtilis*

spores was calculated to be 5046 ±423 and 4030 ±239 respectively, extrapolated to an area of 100 x 100 μm^2, the same area used to collect mid-infrared spectral data. Upon further magnification, SEM images also show the physical properties of the pores on the gold-coated Anodisc™ . Additionally, the SEM confirms that the thickness of the gold layer sputtered onto the Anodisc™ surface is sufficiently thin enough to prevent the blocking of the pores of the membrane. As can be seen in Fig.1, the *B. subtilis* endospores are free of other particulates, such as debris from the growth medium, as a result of the extensive cleanup prior to arrival in our laboratory.[17] Also shown in Fig. 1, the reflected image of the camera can be seen as a darker shadow in the Whatman Anodisc™ illustrating these filters are reflective to visible wavelengths of light.
A total of twelve extinction spectra were collected from a 100 x 100 μm^2 area with a FT-IR microscope at random locations about the Anodisc™ sample. The average of the mid-infrared extinction spectra of *B. subtilis* and PS microspheres on the gold-coated Anodisc™ is shown in Fig. 3, calculated from the recorded reflectance spectra. The bacterial endospore spectrum shows the characteristic bands observed in other infrared studies (Fig. 3A).[18] The most prominent vibrations in mid-infrared spore spectra are due to the amide A at 3300 cm^{-1} (N-H stretching), amide I at 1650 cm^{-1} (carbonyl stretching), and the amide II at 1540 cm^{-1} (a combination of C-N stretching and N-H bending vibrations) protein bands. Other assignments in these typical endospore spectra are lipid C-H stretching in the spectral region from 2850 - 2960 cm^{-1}, and the vibrations at 1443 cm^{-1} and 1388 cm^{-1}, assigned as contributions from calcium dipicolinate (CaDPA), as pyridine ring vibrations and symmetric carboxylate stretching respectively.
It was observed that these absorptions appear at the same frequencies as those from *B. subtilis* spores sampled as dried suspensions on gold-coated microscope slides.[18]
Because bacterial spores and other particles scatter light as a function of the wavelengths of light incident upon them, a sloping baseline is observed. A particulate free Anodisc™ does not exhibit this small extinction that diminishes inversely proportional to increasing wavelength. This apparent absorption can be described by Mie theory for sufficiently small particles like bacterial spores.[16]

Values for C_{abs} and C_{sca} for PS microspheres (1 micron diameter) were calculated from a measured mid-infrared reflectance spectrum, for the 800 – 2000 cm^{-1} spectral range, and literature values for the imaginary refractive index.[19] These values were found to be 16.45 and 1112.3 for C_{abs} and C_{sca} respectively. This spectral range was used in order to ensure Mie theory holds true for particles of this size. The value for C_{abs} was found to be 1.67 times larger than the expected value of 9.87 for a 1 μm sphere for a transmission measurement. Conversely, the scattering constant, C_{sca}, was larger than the calculated value of 204.0 by a factor of 5.45, which is close to 1.67 raised to the fourth power. The difference between the expected and calculated scattering constant is due to the capturing of some of the scattered light by the microscopes optics when operated in reflection mode, otherwise lost during a normal transmission experiment. For an optical system, like the FT-IR microscope used in these studies, with an optical objective having a numerical aperture (NA) equal to 0.58 (equivalent to 35.45°) , assuming the particles are small compared to the wavelength, one can approximate the amount of forward and back scattered light collected by:



(14)

Therefore, approximately 25.5% of the scattered light is collected in these reflection experiments. Thus one would expect that the calculated scattering constant, C_{sca}, would be approximately 75.5% of the ratio of the calculated ($C_{abs\ calc}$) to expected ($C_{abs\ exp}$) absorption constant raised to the fourth power ($[C_{abs\ calc} / C_{abs\ exp}]^4$) times the expected value of C_{sca}. For PS microspheres deposited on these reflective filter substrates, the calculated value of C_{sca} was 71% of this ratio (204.0 x 1.67^4).

When optimal values were found for C_{abs} and C_{sca}, an iterative process was then used to obtain the value for n_∞, the real index offset, which accounts for the inability to collect spectral information at all wavelengths. In the case of the PS microspheres, a value of 1.46 was used to adjust the calculated value of the real index of refraction to match literature values.[19] For *B. subtilis* spores, a value of 1.51 for n_∞ was calculated.

Equipped with these calculated values of C_{abs}, C_{sca} and n_∞, the real and imaginary index (n and k) spectra for the particulate can be calculated. These optical constant spectra were iteratively calculated using a minimization routine in the 800 – 2000 cm^{-1} spectral region for the PS microspheres (Fig. 4). These calculations assumed no prior knowledge of what the k spectrum should be, thus to initiate the optimization, a delta function was utilized by setting wavelengths associated with peak absorbance values to a maximum of one and all others were set to zero. The calculated n and k spectra for spores of *B. subtilis* strain PS832, shown in Fig. 5 and Fig. 6, were consistent with literature values obtained by Querry and Milham, and reported by Gurton et al for *Bacillus globigii*[20] (reclassified as a substrain of *Bacillus atrophaeus*).[21]

Obtaining the optical constant spectra with the use of C_{abs} and C_{sca} in the shorter wavelength region, from 2.5 - 5 μm (2000 - 4000 cm^{-1}), proved difficult due to the change in scattering at these wavelengths. The apparent increase of absorption in these wavelengths appears to deviate from the expected $1/\lambda^4$ behavior. This is due in part by the increase in the collection of the scattered light as the ratio of the particle size to the wavelength of incident light becomes larger. As the particle's size factor increases, the forward to back scattering ratio increases while its angular distribution decreases,[16] therefore more of the scattered light is collected, reducing the scattering extinction.

The absorbance and scattering constants C_{abs} and C_{sca} allow the calculation of their respective cross-sections by Eqs. 7 and 8. The results for PS microsphere are shown in Fig. 7, along with the extinction cross-section (σ_{ext}), the sum of σ_{abs} and σ_{sca}. Fig. 8 shows the results when the constants, C_{abs} and C_{sca}, obtained for PS microspheres were used as a model for spores of *B. subtilis*. The extinction cross-section for the Amide I peak at 1654.7 cm^{-1} was calculated to be 3.6 x 10^{-9} cm^2/endospore, approximately one-

half that reported previously for the Amide A band, but of the same order of magnitude. Similarly, σ_{abs} was found to be 2.9 x 10^{-9} cm^2/endospore for the Amide I band.

Publications and Technical Reports supported under this grant

Two manuscripts are currently in preparation as a result of this effort. The first is::

D. L. Perkins, M. V. Schiza, R. J. Priore, B. V. Bronk, B. Setlow, P. Setlow, and M. L. Myrick "A Simple Method to Extract Optical Constants from Particulates by Infrared Reflectance Microscopy Demonstrated with Polystyrene Microspheres and *Bacillus subtilis* Spores" (manuscript in preparation, 2005).

The second is titled "Calibration curves for scattering and absorbance of particles as a function of surface concentration" by H. Brooke, D.L. Perkins, B.V. Bronk, B. Setlow, P. Setlow and M.L. Myrick.

Participating Scientific Personnel

Michael L. Myrick, Professor, USC Department of Chemistry and Biochemistry (PI).

David L. Perkins, Ph.D. received in July, 2005.

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Appendix I. Figures for Report

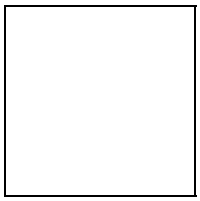

Fig. 1. *Bacillus subtilis* spores filtered across reflective filter media. Photographs of representative gold coated Al_2O_3 membranes before (A) and after (B) *B. subtilis* spores have been filtered onto them.

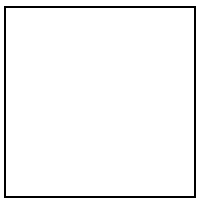

Fig. 2. Polystyrene microspheres filtered across reflective filter media. Images obtained by SEM show that the filtration method yields evenly dispersed particles across the surface. Dark areas in the images are regions of closed pores in the filter media.

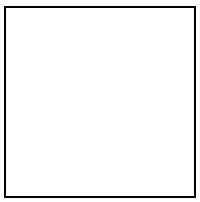

Fig.3. Mid-infrared reflectance spectra of *B. subtilis* spores (A) and polystyrene microspheres (B) filtered across the gold coated Anodisc^{TM}. The spectra show the major absorption peaks associated with bacterial spores and polystyrene respectively. Spectra are shown offset for clarity.

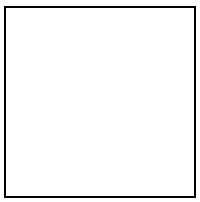

Fig. 4. Calculated real (top and left axis) and imaginary (bottom and right axis) indices of refraction spectra for polystyrene microspheres. Spectra were offset for clarity.

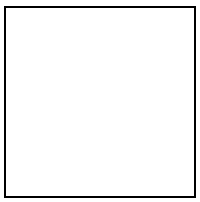

Fig. 5. The real index (n) of refraction (___) for *B. subtilis* spores in the 800 - 2000 cm^{-1} spectral range. Literature values (····) obtained from Querry and Milham for *B. globigii*,[20] are shown here for comparison.

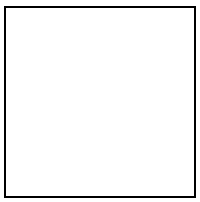

Fig. 6. The imaginary index (k) of refraction (___) for *B. subtilis* spores in the 800 - 2000 cm^{-1} spectral range. Literature values (····) obtained from Querry and Milham for *B. globigii*,[20] shown here for comparison, show good agreement.

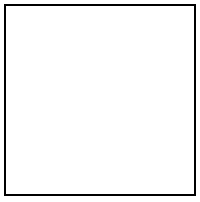

Fig. 7. The cross-sections of absorption (·····), scattering (-----), and total extinction (___) calculated for polystyrene microspheres in the mid-infrared wavelength region from 5 - 12.5 μm (800 – 2000 cm^{-1}).

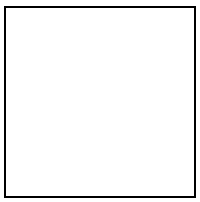

Fig. 8. The cross-sections of absorption (·····), scattering (-----), and total extinction (——) calculated for spores of *B. subtilis* in the mid-infrared wavelength region from 5 - 12.5 μm (800 – 2000 cm^{-1}).